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Interaction between physical activity and smoking on lung, muscle and bone in mice

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Abstract

Physical inactivity is an important contributor to skeletal muscle weakness, osteoporosis and weight loss in chronic obstructive pulmonary disease. However, the effects of physical inactivity, in interaction with smoking, on lung, muscle and bone are poorly understood. To address this issue, male mice were randomly assigned into an active (daily running), moderately inactive (space restriction) or extremely inactive group (space restriction followed by hindlimb suspension to mimic bed rest) during 24 weeks and simultaneously exposed to either cigarette smoke or room air. The effects of different physical activity levels and smoking status and their respective interaction were examined on lung function, body composition, *in vitro* limb muscle function and bone parameters. Smoking caused emphysema, reduced food intake with subsequent loss of body weight, fat, lean and muscle mass but increased trabecular bone volume. Smoking induced muscle fiber atrophy which did not result in force impairment. Moderate inactivity only affected lung volumes and compliance, whereas extreme inactivity increased lung inflammation, lowered body and fat mass, induced fiber atrophy with soleus muscle dysfunction and reduced exercise capacity and all bone parameters. When combined with smoking, extreme inactivity also aggravated lung inflammation and emphysema, and accelerated body and muscle weight loss. This study shows that extreme inactivity, especially when imposed by absolute rest, accelerates lung damage and inflammation. When combined with smoking, extreme inactivity is deleterious for muscle bulk, bone and lungs. These data highlight that the consequences of physical inactivity during the course of COPD should not be neglected.

Keywords

COPD, muscle function, muscle atrophy, hindlimb suspension, cigarette smoke exposure

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Introduction

Chronic obstructive pulmonary disease (COPD) is accompanied by several comorbidities such as skeletal muscle dysfunction (1), body weight loss (2) and osteoporosis (3). In particular, skeletal muscle dysfunction is of major concern since it contributes to decreased functional capacity (4), poor quality of life (5), increased healthcare utilization (6) and even mortality (7, 8), independently of lung function (9). Skeletal muscle dysfunction in COPD is characterized by reduced muscle strength and endurance and is common in the lower limb muscles, such as the quadriceps muscle. Abnormalities within the locomotor muscles include a fiber shift towards a more glycolytic profile, reduced number of capillary contacts, fiber atrophy and muscle mass loss (1). Furthermore, the reduction in fat-free mass index, which worsens with disease severity, is associated with osteoporosis (10) and is a strong predictor for mortality (11). Several factors, such as physical inactivity, oxidative stress, and systemic inflammation are important contributors to these comorbidities (1, 12).

In patients with COPD, shortness of breath experienced during daily life activities contributes to a further reduction in physical activity levels because of the unpleasant sensation caused by exertion. Physical inactivity, which is common in patients with COPD (13), occurs early in the development of the disease (14) and worsens dramatically during hospitalization for exacerbations (15). The fact that high levels of regular physical activity are associated with a lower lung function decline and lower risk of developing COPD in active smokers (16) and in smoking animals (17), suggests a causal involvement of physical inactivity in COPD progression.

Avoiding physical activity due to breathlessness makes patients with COPD more sedentary and deconditioned. Muscle deconditioning is characterized by reduced muscle strength and mass, fiber atrophy and loss of type I fibers (18) as well as osteoporosis (19). These are all features that are also observed in patients with COPD (1). Apart from deconditioning, as a

consequence of physical inactivity, cigarette smoking also plays an important role in the loss of body weight (20), skeletal muscle dysfunction (21) and osteoporosis (22), potentially via systemic inflammation and oxidative stress.

Currently, it is unclear whether physical inactivity can causally affect lung function and structure in addition to its impact on muscle and bone. The respective effects of physical inactivity and cigarette smoking on lung, muscle and bone are also unclear and whether these effects would be aggravated when physical inactivity is combined with cigarette smoking has not yet been determined.

Therefore, we aim at examining the respective impact of physical inactivity, cigarette smoking and their combined effect on lung function, skeletal muscle function and bone, in order to highlight their potential role in lung function decline and the development of comorbidities in patients with COPD. We hypothesized that 24 weeks of physical inactivity or cigarette smoking would affect lung, muscle and bone and that these effects would be aggravated when both are combined. To address this issue, active mice, daily running on a treadmill and having free access to a cage wheel, were compared to mice made moderately inactive through cage space restriction and to severely inactive mice obtained by combining cage space restriction with short-term hindlimb suspension in order to mimic bed rest as during hospitalization. Simultaneously, these mice were daily exposed to either cigarette smoke or room air for 24 weeks. The development of lung disease (function and histology), the impact on muscle function and structure (contractility, mass, histology and exercise capacity), the changes in body composition (fat and lean and bone mass) and in bone parameters (volume and thickness and cross-sectional area) were assessed at 24 weeks.

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Abbreviations:

COPD	Chronic Obstructive Pulmonary Disease
BAL	Broncho-Alveolar Lavage
CSA	Cross-Sectional Area
DEXA	Dual X-ray Absorptiometry
EDL	Extensor Digitorum Longus
Lo	Optimal length
MEC	Maximal Exercise Capacity
MHC	Myosin Heavy Chain
PBS	Phosphate Buffered Saline

Material and Methods

Study Design (Figure 1)

Male C57Bl/6 mice were randomly divided into an active (Ac), moderately inactive (I) or extremely inactive (E) group. Simultaneously, mice within each group were daily exposed to ambient air (Ac n=12; I n=7; E n=13) or cigarette smoke (Ac n=12; I n=7; E n=13) for 24 weeks. Active mice, performing daily regular activity by running on a treadmill and through free access to a cage wheel, served as a control group. Those mice were allowed to rest for at least 1 hour before exposure to cigarette smoke or ambient air. Moderate inactivity was induced by cage space restriction. Extreme inactivity was induced by 21 weeks of space restriction followed by 3 weeks of hindlimb suspension. Body weight and food intake were measured weekly. All experimental procedures were approved by the Ethical Committee of Animal Experiments of KU Leuven.

Maximal exercise capacity (MEC) test

The MEC test was assessed at baseline and after 24 weeks. MEC was defined as the maximal speed reached by each animal.

Measurements after 24 weeks at sacrifice:

Wire-hang test

Mice were placed on a grid, which was then inverted and time until falling from the grid (latency-to-fall time) was recorded.

Body composition

Mice were anaesthetized with a mixture of xylazine and ketamine to assess fat, lean and bone mass using a DEXA scan.

Lung assessments

Tracheotomized mice were placed in a body plethysmograph to measure total lung capacity and lung compliance.

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To assess inflammation in the broncho-alveolar lavage fluid, lungs were lavaged 4 times with Dulbecco's phosphate buffered saline (PBS). Pellets obtained after centrifugation were dissolved in PBS for total and differential cell counting.

To examine lung structure, sagittal sections of the heart-lung block were stained with hematoxylin and eosin to measure air space enlargement, through the mean linear intercept.

Muscle assessments

Left and right soleus, EDL and gastrocnemius muscles were weighted and mass sum was used for analysis. Maximum twitch and tetanic force (300Hz) and force-frequency relationship were assessed *in vitro* in the EDL and soleus muscle. Twitch characteristics (half relaxation time and time to peak tension) were calculated. Muscle cross-sectional area (CSA) was calculated as bundle mass divided by optimal length and specific density.

Serial cross-sections of soleus and EDL muscle were stained to determine structural changes and dimensions and proportions of the different muscle fibers.

Bone assessments

Bone CSA, cortical thickness and trabecular bone volume of the tibia were assessed using μCT. Tibia length was measured.

Statistics

Comparisons between the groups were performed using a 2-Way ANOVA (SAS 9.3) in order to assess the effect of smoking status and physical activity levels, with the inclusion of an interaction term between both variables. To assess the effect of body weight and food intake, a 2-Way ANOVA was performed with time and group being the independent variables. A Tukey-Kramer *post hoc* test was used for multiple comparisons. Spearman's rho was used to assess correlations.

More details in online supplement.

Results

Lung assessments

Lung function

Compared to mice breathing room air, smoking induced emphysema as indicated by an increase in total lung capacity (+11%, $p<0.05$; Figure 2A) and lung compliance (+13%, $p<0.01$; Figure 2B). Total lung capacity and lung compliance were the lowest in the moderately inactive mice compared to others, independently of smoking status. There was no interaction effect between physical activity levels and smoking on lung function.

Lung histology

Smoking caused air space enlargement as shown by increased mean linear intercept (+17%, $p<0.0001$). Interestingly, mean linear intercept was higher in the extremely inactive mice (smoking and air exposed) compared to the active mice ($p<0.01$; Table 1). There was, however, no interaction effect between physical activity levels and smoking.

Lung inflammation

As expected, smoking increased total cell count in broncho-alveolar lavage (BAL) fluid (x2, $p<0.001$), number of macrophages (x1.5, $p<0.01$) (data not shown) and neutrophils (x12, $p<0.0001$; Table 1) compared to mice exposed to air. Number of neutrophils was higher in extremely inactive mice compared to active mice (x3, $p<0.01$). There was an interaction effect between extreme inactivity and smoking on the enhanced number of neutrophils ($p<0.05$; Table 1). Smoking shifted the percentage of BAL cells towards neutrophils ($p<0.0001$) and, although the interaction term was not significant ($p=0.1087$), this shift was more pronounced in the moderately inactive (+9%, $p<0.001$) and extremely inactive (+9%, $p<0.0001$) mice compared to the active mice (+5%, $p<0.05$). Number of lymphocytes was similar between smoking and air exposed mice (data not shown).

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Muscle and body composition

Food intake

Smoking slightly reduced food intake. Up to week 21, inactivity, consisting in space restriction for all inactive mice, reduced food intake immediately (about 25%), and independently of smoking, compared to active mice (Figure 3A) and this effect remained over time. During hindlimb suspension, food intake of the extremely inactive mice (both smoking and air exposed) increased to a level similar to that of the active mice (Figure 3B).

Body weight and body composition

Up to week 21, body weight was lower in the smoking groups compared to the mice breathing room air, with no effect of physical activity (Figure 3C). After hindlimb suspension, body weight decreased with extreme inactivity (-10% from the second week of hindlimb suspension on, $p<0.0001$) compared to others (Figure 3D). At the end of the study, body weight gain was 12-15% lower in the smoking groups compared to the room air groups ($p<0.0001$) and active mice had the highest body weight gain (+23%) compared to moderately inactive (+16%, $p<0.05$) and extremely inactive mice (+10%, $p<0.0001$; Figure 4A). As a consequence, body weight gain of the extremely inactive smoking mice was the lowest (+3.5%) compared to other groups (Figure 4A), but there was no interaction effect.

Smoking reduced fat mass (mainly central fat mass) (-15%, $p<0.0001$; Figure 4B) and lean mass (-12%, $p<0.0001$; Figure 4C). Fat mass was the highest in the moderately inactive mice ($p<0.001$) and the lowest in extremely inactive mice ($p<0.0001$). As a consequence, fat mass of the extremely inactive smoking mice was the lowest. There was no interaction effect between physical activity levels and smoking on body composition parameters.

Muscle mass (Table 2)

Muscle mass was lower in the smoking groups compared to mice breathing room air (EDL: -9%, $p=0.0001$; soleus: -3%, $p=0.0645$; gastrocnemius: -10%, $p<0.0001$). Moderate inactivity

did not affect muscle mass, while soleus and gastrocnemius mass were significantly reduced with extreme inactivity (-41% and -17%, respectively ($p<0.0001$)). There was no interaction effect.

In vitro contractile properties

Smoking did not affect absolute and specific force of the soleus and EDL muscle (Figure 5 and Table 2). Physical inactivity did not exert any effect on EDL force. Extreme inactivity reduced absolute (-62%) and specific force (-35%) of the soleus muscle but only at high frequencies ($p<0.001$; Figure 5B and Table 2, specific force data), with no further effect of smoking. Neither smoking, nor physical inactivity did alter half relaxation time and time to peak tension in either muscle (Table 2). There was no interaction effect.

Muscle histology

No structural abnormalities in the soleus and the EDL muscle were found in any of the groups. Smoking reduced dimensions of all fiber types in the soleus and EDL muscles ($p<0.01$; Figure 6). Extreme inactivity reduced fiber dimension of the soleus muscle ($p<0.0001$; Figure 6). There was no interaction effect between physical activity levels and smoking.

Smoking induced a shift towards a more glycolytic profile (EDL type IIB: +7%, $p=0.04$, and soleus type IIX: +36%, $p=0.002$) while both moderate and extreme inactivity induced a shift towards an oxidative profile in the soleus (+13%, $p=0.001$).

Maximal exercise capacity test and latency-to-fall time

Compared to baseline values, running speed at 24 weeks was increased in active and moderately inactive mice and decreased in extremely inactive mice. As a consequence, speed was higher in active (pooled values smoking and air exposed: +9%) and moderately inactive mice (pooled values: +12%) compared to extremely inactive mice (pooled values: -9%, $p<0.0001$), with no effect of smoking (Online Supplement Figure E1). Latency-to-fall time

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was the highest in the active mice compared to others ($p<0.01$) and the lowest in the extremely inactive mice ($p<0.05$), with no effect of smoking (Online Supplement Figure E2).

Bone assessments

Neither smoking nor physical inactivity altered mean cross-sectional area of the tibia (pooled values 1.87±0.15 mm²). Smoking increased bone mass (+7%, $p<0.05$; Figure 7A) as shown by increased trabecular bone volume of the tibia (+25%, $p<0.0001$; Figure 7B). In addition, smoking resulted in a 3% shorter tibia length (pooled values smoking: 18.6±0.6 mm versus air exposed 19.2±1.2 mm, $p<0.05$), independently of activity levels. Only extreme inactivity affected all bone parameters by reducing bone mass (-9%; Figure 7A), trabecular bone volume (-23%; Figure 7B) and cortical thickness (-6%; Figure 7C), compared to others ($p<0.0001$). In line with the latter, the inner perimeter of the cortical bone is increased in the extremely inactive mice (+4%, $p=0.09$). As a consequence, extremely inactive mice had worse bone quality than others, with no effect on bone length. There was no interaction effect between physical activity levels and smoking on bone parameters.

Correlations muscle and bone parameters

Cross-sectional area (CSA) of the soleus muscle was positively correlated with cortical bone thickness ($r=0.73$, $p<0.0001$) and trabecular bone volume ($r=0.51$, $p=0.0051$). There was no correlation with EDL CSA and these parameters. The correlations in the soleus muscle remained present when analysis was done in the smoking group or in the extremely inactive group alone.

Discussion

Our data support the concept that both smoking and physical inactivity have an independent impact on lung, muscle function and bone. Smoking induced emphysema, caused body weight loss through the loss of fat and lean mass, but did not affect muscle contractility, albeit the presence of muscle mass loss and fiber atrophy. Moderate inactivity solely reduced lung volume and compliance, whereas extreme inactivity induced lung damage and lung inflammation and was associated with loss of body weight, muscle and bone mass, despite appropriate food intake. Extreme inactivity also caused fiber atrophy and impaired contractility of the soleus muscle. When combined with smoking, it resulted in more detrimental effects on the lung, body and muscle mass.

As expected, smoking caused emphysema (23), reduced food intake (24, 25) and lowered body weight (23, 26-29). While reduced food intake could only partially explain the lowered body weight, as previously reported (24), loss of fat mass (28) and reduced lean mass and body growth also contribute to body weight loss. In line with other observations, smoking induced fiber atrophy (30, 31) and resulted in the loss of muscle mass (32) whilst it did not affect muscle contractility (23). Additionally, the shift towards a more glycolytic profile in both muscles, as shown before (23, 33, 34), did not result in impaired maximal exercise capacity, which is in agreement with other mouse studies (17, 34) but contrasting with the data in patients with COPD (35). Discrepancies may be due to the fact that the mouse model of cigarette smoke exposure induced a mild form of human lung disease. Intriguingly, total bone mass was increased with smoking in the current study, while in humans, smoking is a known risk factor for bone loss and increased risk of fractures (36). In animals, the effects of cigarette smoke exposure on bone are controversial (37, 38) and the timing of bone loss may be associated with the dose and duration of smoke exposure (39).

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Although moderate inactivity lowered food intake and body weight gain in the current study, it did not alter muscle mass or contractility and bone parameters. However, compared to other groups, total lung capacity and lung compliance were lower in the moderately inactive mice, independently of smoking. This could not be explained by reduced body weight gain, as tibia length was not affected by physical inactivity. Neither is this explained by differences in body weight since moderately inactive and extremely inactive mice showed similar body weight at sacrifice. As expected from data in human studies (40), moderate inactivity induced a shift towards higher fat mass, and therefore a proportionally lower lean mass. Redistribution of fat in the thorax and the abdomen may therefore explain the reduced lung volumes and compliance. A disproportional fat mass may also explain why the moderately inactive mice spent less time hanging on the grid compared to active mice. When combined with smoking, moderate inactivity further reduced body weight gain, even though food intake was not changed compared to moderately inactive mice breathing room air. No detrimental effects on muscle and bone were observed, even though lung inflammation was slightly more pronounced in this group, as shown by a higher percentage of neutrophils compared to smoking active mice. Together, our data suggest that moderate inactivity, mimicking a sedentary situation, has no major impact on muscle and bone, but alters lung function through alterations in body composition towards more fat mass.

Interestingly, extremely inactive mice had more lung damage and inflammation. It is yet not clear how extreme inactivity is enhancing lung disease but it is tempting to relate these findings to an excess of oxidative stress. A similar phenomenon is observed in skeletal muscles (41, 42) in which physical inactivity, through oxidative stress, can lead to activation of inflammatory (43, 44) and proteolytic pathways (41, 42). Otherwise, enhanced lung inflammation in extremely inactive mice might be related to the fact that hindlimb suspension caused a redistribution of body fluids towards the upper body parts. Indeed, due to hanging at

a 30° head down position, coughing is impaired, leading to reduced clearance of inflammatory cells (45). Extreme inactivity resulted in body weight loss as previously observed (46) but this was not due to a reduction of food intake that, in fact, increased during the period of hindlimb suspension towards the levels of active mice (46). Loss of body weight was accompanied with loss of soleus and gastrocnemius mass and a pronounced loss of fat mass, which are likely due to hypermetabolism (47). As expected (32), loss of muscle mass, fiber atrophy and muscle dysfunction, induced by hindlimb suspension, was greater in the weight bearing muscles of the ankle, such as the soleus and gastrocnemius, than in the non-weight bearing muscles, such as the EDL (48). These observations are in agreement with the effects of muscle deconditioning, a direct consequence of physical inactivity. In addition, extreme inactivity reduced both cortical thickness and trabecular bone volume, which is in line with previous studies on hindlimb unloading (49). Finally, loss of body weight and muscle mass will certainly contribute to the lower maximal exercise capacity and latency-to-fall time seen with extreme inactivity. Taken together, these data are particularly relevant as they show that extreme inactivity, as often observed in hospitalized patients, was deleterious for lung, skeletal muscles and bone. Clearly, bed rest or hindlimb suspension not only appears to be an important contributor to skeletal muscle weakness and osteoporosis, independently of smoking, but it may also favor lung damage and inflammation.

Importantly, when extreme inactivity was combined with smoking, lung damage and inflammation aggravated. Actually, mean linear intercept, used as an index of lung morphological emphysema, and neutrophil content in broncho-alveolar lavage fluid were the highest under these circumstances. These observations suggest that extreme inactivity, for instance by bed rest, may aggravate the development of smoke-induced lung disease. These findings are in line with previous mouse studies, showing that regular aerobic physical training of moderate intensity attenuated the development of smoke-induced lung disease, by

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lowering the levels of oxidative stress (17). They also corroborate epidemiological findings in humans indicating that moderate to high levels of regular physical activity lowered the risk of COPD in persistent smokers (16) and associated with lower levels of oxidative stress (50). Further studies are warranted to unravel the mechanisms by which extreme inactivity accelerates lung damage and inflammation when combined with smoking.

In conclusion, our study shows that cigarette smoking and physical inactivity have independent noxious effects on lung, muscle and bone. Our data suggest that extreme inactivity, which may be imposed during hospitalization, is an important factor in the deterioration of human COPD.

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Tables

Table 1. Lung assessments. Mean linear intercept and number of neutrophils in the lung. Values are expressed as means ± SD.

	AIR			SMOKING		
	Active	**Moderately inactive**	**Extremely inactive**	**Active**	**Moderately inactive**	**Extremely inactive**
Mean linear intercept (µm)	48 ± 4	50 ± 2	51 ± 2 #	56 ± 3 ***	57 ± 3 ***	61 ± 5 *** #
Neutrophils (x 10 4 cells / ml BAL)	0.02 ± 0.02	0.04 ± 0.05	0.10 ± 0.11 #	0.36 ± 0.17 ***	0.49 ± 0.37 ***	1.01 ± 0.60 *** #

*** $p<0.0001$ versus air; # $p<0.01$ versus Active

Interaction between extreme inactivity and smoking on number of neutrophils ($p<0.05$)

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Table 2. Muscle characteristics. Mass of soleus, EDL and gastrocnemius muscle, maximal tetanic force and twitch characteristics of EDL and soleus muscle. Values are expressed as means ± SD.

	AIR			SMOKING		
	Active	**Moderately inactive**	**Extremely Inactive**	**Active**	**Moderately inactive**	**Extremely inactive**
Mass (mg)						
EDL	24.7 ± 2.5	26.1 ± 1.0	23.6 ± 2.6	22.7 ±2.4 *	22.4 ± 1.9 *	21.3 ± 2.4 *
SO	22.3 ± 2.8	22.7 ± 1.9	12.6 ± 1.7 †	20.1 ± 2.2 ‡	20.9 ± 2.0 ‡	12.7 ± 0.9 ‡†
GA	296.1 ± 24.2	303.7 ± 22.3	241.9 ± 22.7 †	259.5 ± 24.0 *	264.3 ± 18.5 *	218.7 ± 24.2 *†
Tetanic Force (g/cm²)						
EDL	5211 ± 889	4902 ± 371	5234 ± 936	4771 ± 947	4698 ± 957	3950 ± 1360
SO	3427 ± 223	3179 ± 828	1888 ± 632 †	2897 ± 822	3358 ± 517	2110 ± 812 †
Half relaxation time (ms)						
EDL	13 ± 5	13 ± 6	13 ± 5	15 ± 6	14 ± 4	14 ± 4
SO	21 ± 6	21 ± 3	18 ± 8	21 ± 5	22 ± 1	20 ± 6
Time to peak tension (ms)						
EDL	15 ± 5	13 ± 4	15 ± 4	13 ± 5	13 ± 5	12 ± 5
SO	19 ± 2	20 ± 6	20 ± 4	20 ± 4	22 ± 4	18 ± 4

* $p<0.0001$ versus Air; † $p<0.0001$ versus others; ‡ $p=0.0645$ versus Air

Figure legends

Figure 1. Study design. All mice were divided randomly into 3 groups of physical activity: active (Ac – 24 weeks of treadmill running and cage wheel), moderately inactive (I – 24 weeks of space restriction) or extremely inactive (E – 21 weeks of space restriction followed by 3 weeks of hindlimb suspension). Afterwards, within each group, mice were randomly divided into a group that was exposed to cigarette smoke (smoking) or room air (air). The total period of the study was 24 weeks. Body weight (BW) and food intake (FI) were measured weekly, while lung, muscle and bone parameters were assessed after 24 weeks. Maximal exercise capacity (MEC) test was assessed at baseline and after 24 weeks.

Figure 2. Effects of cigarette smoke exposure and different physical activity levels on lungs. Smoking increased total lung capacity (A) and lung compliance (B) in active (Ac), moderately inactive (I) and extremely inactive (E) mice. Moderate inactivity reduced total lung capacity and lung compliance. Values are expressed as means ± SD. * $p<0.05$ versus Air; # $p<0.05$ versus others

Figure 3. Effects of cigarette smoke exposure and different physical activity levels on food intake and body weight over time. Smoking (open symbols) had mild, non-significant effects on food intake ((A) until week 20 and (B) week 20-24) and on body weight over time ((C) until week 20 and (D) week 20-24) in active (circles), moderately inactive (squares) and extremely inactive (triangles) mice, compared to mice exposed to breath room air (closed symbols). Black and grey arrows indicate start of smoking or breathing room air and hindlimb suspension, respectively. Values are expressed as means ± SD. § $p<0.0001$ E versus others; §§ $p<0.001$ I versus others

Figure 4. Effects of cigarette smoke exposure and different physical activity levels on body weight gain and body composition. Body weight gain (A), fat mass (B) and lean mass

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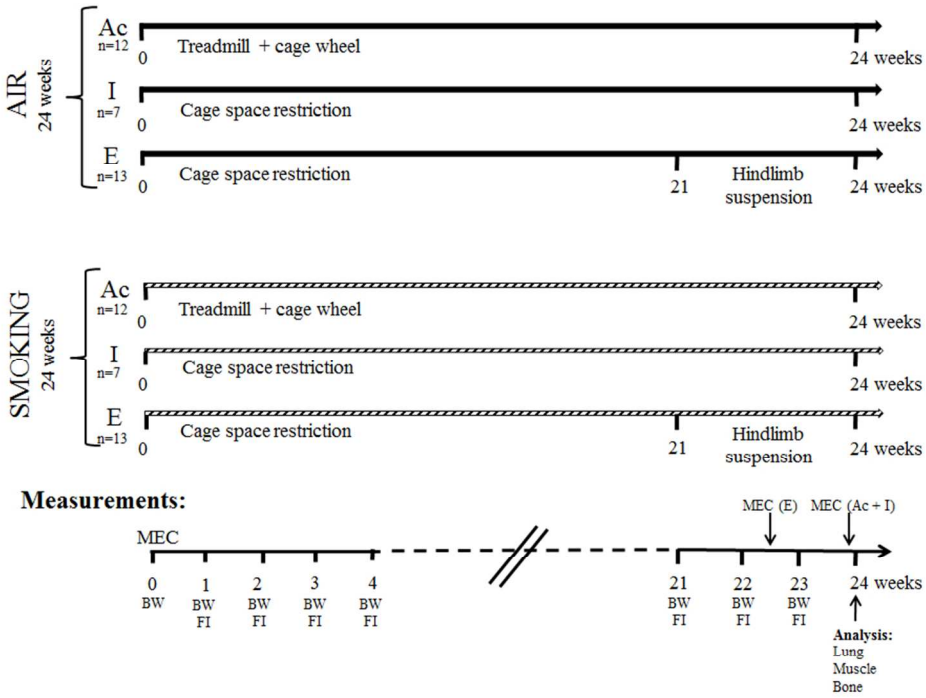
(C) were reduced with smoking in active (Ac), moderately inactive (I) and extremely inactive (E) mice. Moderate and extreme inactivity reduced body weight gain, and fat mass was the highest in moderately inactive, and the lowest in extremely inactive mice. Values are expressed as means ± SD. *** $p<0.0001$ versus Air; # $p<0.05$ I versus A; ## $p<0.0001$ E versus A (fig A) or others (fig B)

Figure 5. Effects of cigarette smoke exposure and increasing physical inactivity levels on muscle function. Smoking (open symbols) did not affect force-frequency curve of EDL (A) and soleus (B) muscles in active (circles), moderately inactive (squares) and extremely inactive (triangles) mice compared to ambient air (closed symbols). Values are means ± SD. # $p<0.001$ E versus others

Figure 6. Effects of cigarette smoke exposure and increasing physical inactivity levels on muscle histology. Fiber dimensions of EDL (A) and soleus (B) muscles were reduced with smoking, while all fiber dimensions in the soleus muscle were reduced in the extremely inactive (E) mice compared to active (Ac) and moderately inactive (I) mice. Values are means ± SD. * $p<0.01$ Smoking versus Air; ## $p<0.0001$ E versus others.

Figure 7. Effects of cigarette smoke exposure and different physical activity levels on bone parameters. Smoking increased bone mass (A) and trabecular bone volume (B). Extreme inactivity reduced trabecular bone volume (B) and cortical thickness (C) of the tibia in active (Ac), moderately inactive (I) and extremely inactive (E) mice. Values are means ± SD. # $p<0.0001$ E versus others; * $p<0.05$; § $p<0.001$ E versus A; *** $p<0.0001$ versus Air.



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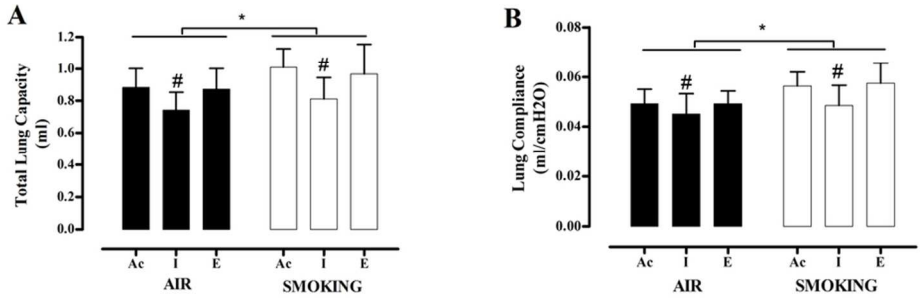

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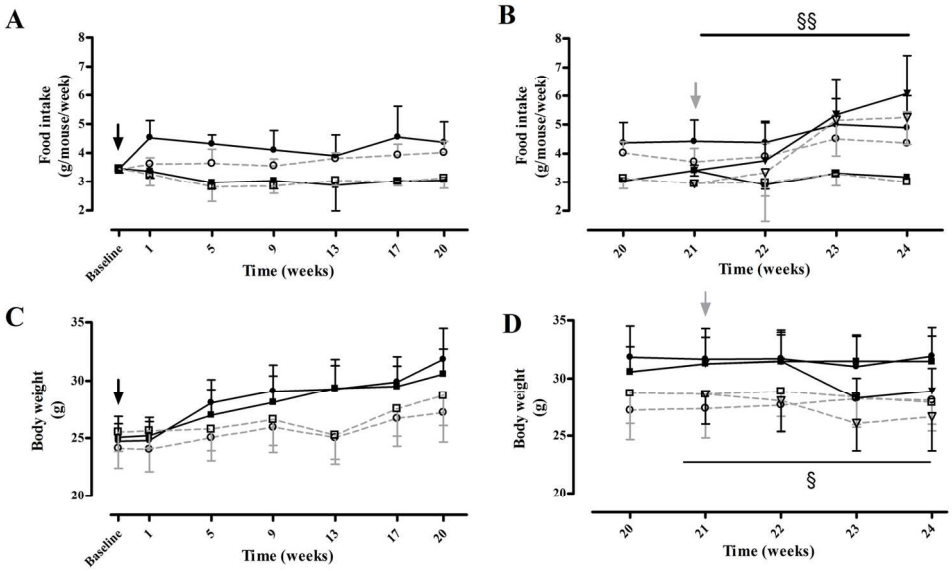

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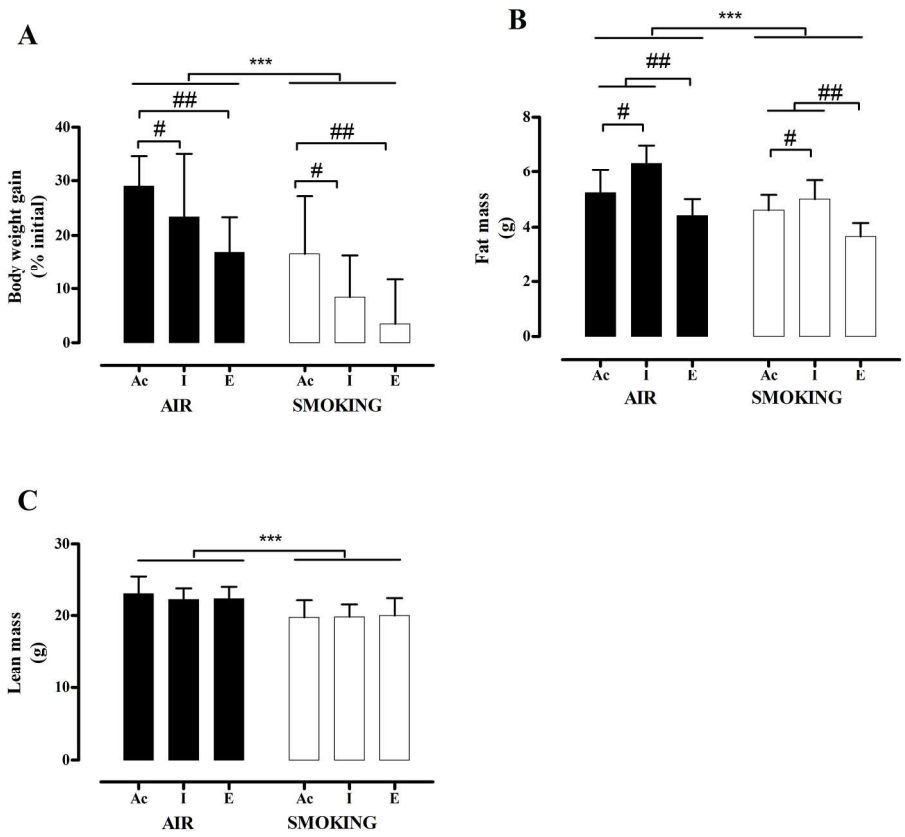

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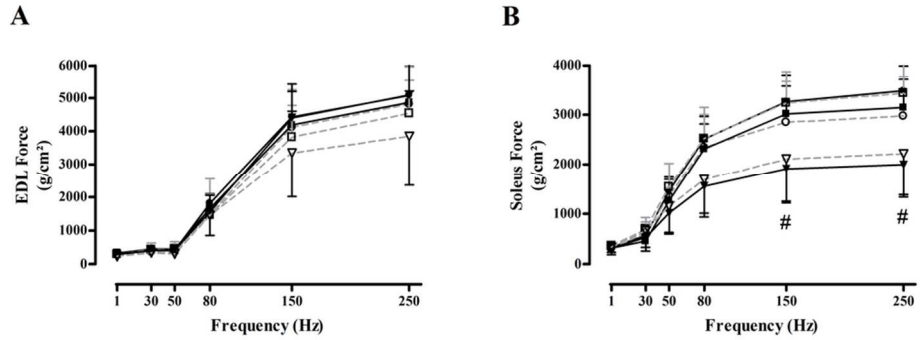

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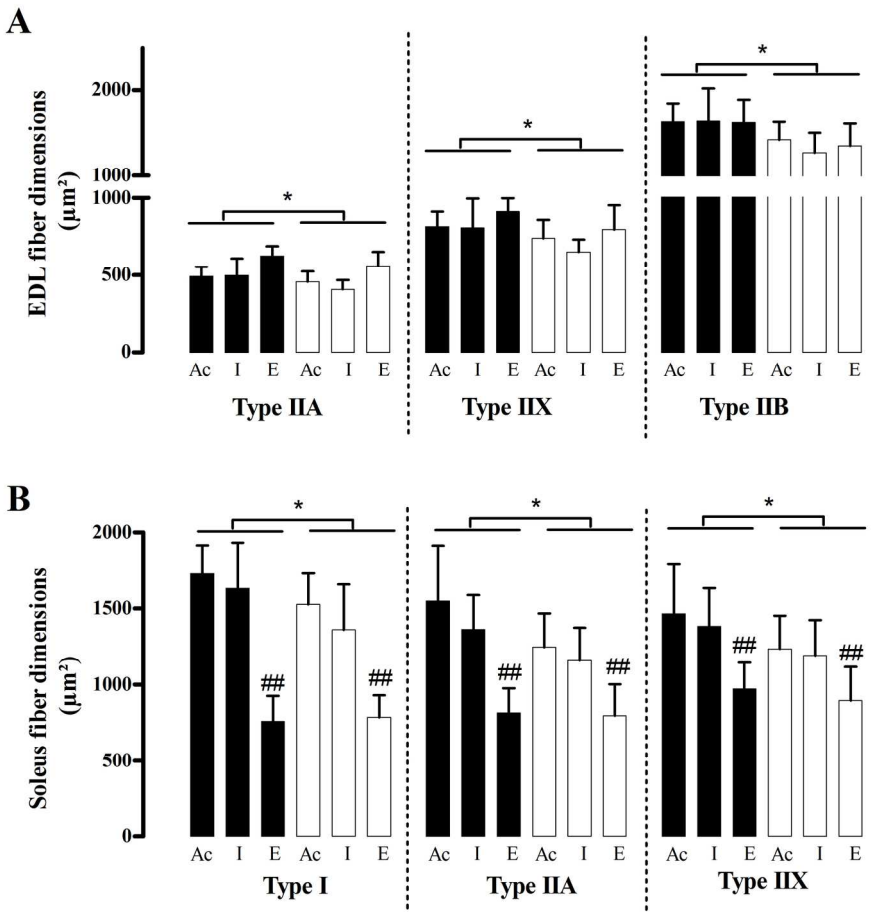

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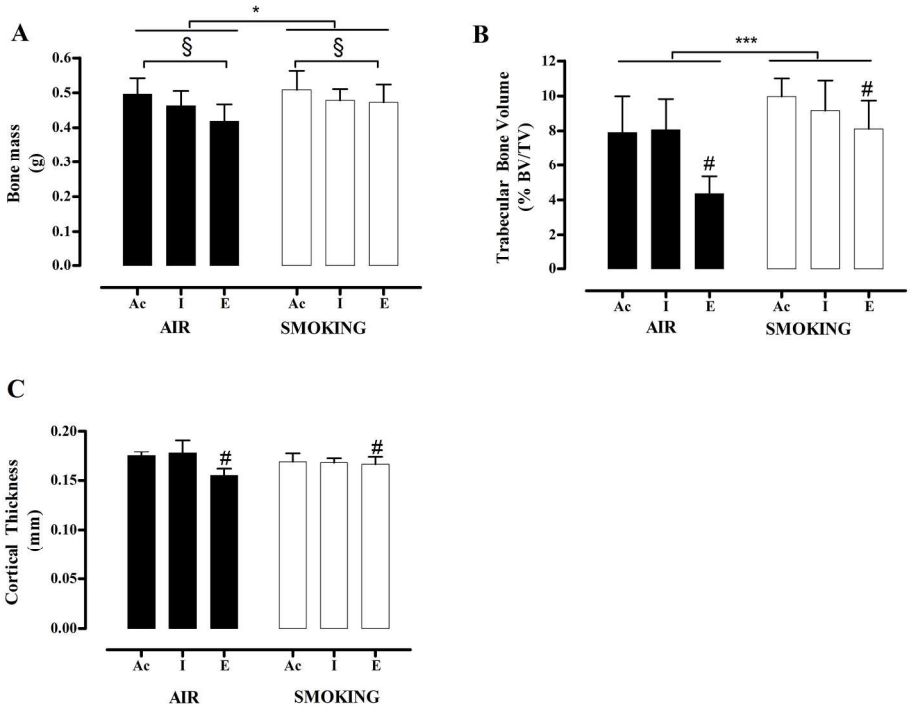

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Online Data Supplement

Material and Methods

Study Design

Sixty-four male C57Bl/6 mice (25±2g, 8 weeks old, Harlan, The Netherlands) were randomly divided into different physical activity level groups: 1) Active (Ac, n=24), 2) moderately Inactive (I, n=14) or 3) Extremely Inactive (E, n=26) mice. Mice within each group were at the same time daily exposed to either breathe ambient air (air: Ac (n=12); I (n=7); E (n=13)) or cigarette smoke (smoking: Ac (n=12); I (n=7); E (n=13)). Activity levels and exposure to cigarette smoke or room air started concomitantly and for a total duration of 24 weeks (Figure 1). Active mice served as control mice in this study. Mice were exposed to 4 cigarettes, 2 times a day, 5 days a week or to room air for the same duration via a nose-only exposure system (SCIREQ, USA) (1). Particle density was measured daily (Microdust Casella). Active mice were performing regular activity (treadmill running and cage wheel) as described hereafter. Moderately inactive mice were housed in space restricted cages (20 cm^2 instead of 70 cm^2/mouse) (2) and extremely inactive mice were housed in space restricted cages for 21 weeks, followed by a hindlimb suspension for 3 weeks. Hindlimb suspension was induced by tail traction with the animal in a 30° head-down position, allowing the mice to rotate 360°, with access to food and water *ad libidum* (3). Body weight and food intake were recorded weekly. All experimental procedures were approved by the Ethical Committee of Animal Experiments of KULeuven.

After 24 weeks, lung, muscle, body and bone assessments were performed as described hereafter.

Maximal exercise capacity test, and wire-hang test

Maximal exercise capacity test was assessed at baseline and after 24 weeks for the active and moderately inactive groups, and at day 10 of the hindlimb suspension for the extremely inactive group. Prior to the test at baseline, mice were accustomed to run on the treadmill for 3 days (0% incline, 10 minutes at 3m/min, and 10 minutes at 6m/min). Maximal exercise capacity test consisted in a 5 minute warming up (0% incline, 3m/min), followed by a run with increasing speed by 1m/min every minute until animal exhaustion was achieved, defined as the inability to run after 10 gentle mechanical stimuli (based on (4)). Running speed was recorded. Maximal exercise capacity was defined as the maximal speed reached by each animal.

Active mice were performing daily regular activity by running on a treadmill at 65-70% of their maximal exercise capacity for 30 minutes, 5 days a week, and by running voluntary in a wheel placed inside their cage. After running on the treadmill, active mice were allowed to rest for at least 1 hour, before being exposed either to cigarette smoke or to ambient air.

Before sacrifice, whole-body strength was assessed by using a wire-hang test to measure latency-to-fall time. Therefore, mice were positioned on a grid placed horizontally with the four limbs grabbing the grid. The grid was then inverted and time until the mice fall from the grid was recorded (5).

Measurements after 24 weeks at sacrifice:

Lung assessments

Pulmonary function measurements

Twenty-four hours after exposure to the last cigarette or to ambient air, mice were intraperitoneally anaesthetized with a mixture of xylazine (8.5 mg/kg, Rompun®, Bayer, Belgium) and ketamine (13 mg/kg, Anesketin®, Eurovet, Belgium). A tracheotomy was

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performed and mice were placed in a body plethysmograph (Buxco©-Force Pulmonary Maneuvers®, USA) to measure total lung capacity and lung compliance, as previously described (1).

Cellular composition of the broncho-alveolar lavage (BAL)

The lungs were lavaged 4 times with 1ml ice cold Dulbecco's phosphate buffered saline (PBS). After centrifugation (1000g, 10 minutes, 4°C), pellets were dissolved in 1ml PBS for total and differential cell counting. Total cell counting was performed using a Bürker hemocytometer. For differential cell count, cytospins (Shandon) were colored with May-Grünwald-Giemsa staining and 300 cells per mouse were counted to determine the number of macrophages, neutrophils and lymphocytes.

Histopathology of the lungs

The heart-lung block was fixed in 6% paraformaldehyde at a constant hydrostatic pressure of 25 cmH_2O for 24 hours, and embedded in paraffin. Sagittal sections were stained with hematoxylin and eosin (H&E) to evaluate air space enlargement, as assessed by the mean linear intercept. Mean linear intercept was measured in 15 randomly selected fields per slide at a 200x magnification and calculated as the total length of the grid lines × random fields divided by the sum of the alveolar intercepts (6).

Body and muscle assessments

Body composition

To assess body composition, the dual X-ray Absorptiometry (DEXA) scan was used. Total tissue mass, fat percentage and bone mass were measured using the PIXImus mouse densitometer (Luner Corp., Madison, WI, USA) and lean mass, lean mass percentage and total fat mass were calculated.

Muscle mass, in vitro muscle contractile properties and histology

Gastrocnemius, soleus and extensor digitorum longus (EDL) of the left and right hindlimbs were weighted. The sum of left and right muscle mass was used for analysis and data are presented as such in the results section.

The left soleus and EDL muscle were used to assess their *in vitro* contractile properties, as described previously (1, 7). Briefly, contractility was measured *in vitro* at 37°C using a temperature-controlled organ bath and stimulating electrodes. Optimal muscle length (Lo) for peak twitch force was established and the following measurements were performed at Lo: 1) maximum twitch force, 2) maximal tetanic force (300Hz), 3) force-frequency relationship at 1, 30, 50, 80, 150 and 250Hz. Twitch characteristics (half relaxation time and time to peak tension) were calculated. Lo was measured as well as muscle weight. Muscle CSA was calculated as weight divided Lo and specific density. Force in gram (absolute force) and corrected by muscle cross-sectional area (CSA) (specific force) were used for analysis.

Right soleus and EDL muscles were quickly frozen in isopentane cooled in liquid nitrogen and stored at -80°C. Sections of 5µm thickness were stained with H&E to determine structural changes. To determine the dimensions and proportions of the different muscle fibers, serial cross-sections were blocked (10% normal goat serum in PBS) for 1 hour and then incubated at room temperature for 2 hours with a primary antibody cocktail, specific to laminin (ab11575, Abcam), myosin heavy chain (MHC)-I (BA-F8), MHC-IIA (SC-71) and MHC-IIB (BF-F3) in blocking buffer. All MHC antibodies were purchased from Developmental Studies Hybridoma Bank. After washing the slides 3 times for 5 minutes in PBS, they were incubated for 1 hour with a secondary antibody cocktail (Alexa Fluor 532, Alexa Fluor 350, Alexa Fluor 488, Alexa Fluor 555, Life Technologies) in blocking buffer. The sections were mounted with coverslips with ProLong® Gold antifade reagent. Using a computerized image system (CellSens, Olympus) about 150 fibers per muscle were used to determine dimensions and

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proportions of the different fibers. The relative contribution of the different fiber types to total cross-sectional area of the muscle was also calculated.

Bone assessments

The left tibia was fixed in Burckhardt medium for 24 hours and then washed and stored in ethanol 100%. Prior to scanning, the bone was positioned in a sample holder filled with distilled water. *Ex vivo* µCT analysis was performed using the high resolution Skyscan 1172 System (50kV and 200µA and a 0.5 mm Al filter) according to the Guidelines (8). Cortical thickness and trabecular bone volume as well as mean cross-sectional area were calculated. Afterwards, tibia length was measured.

Statistical analysis

Statistical analysis was performed using a SAS 9.3 Statistical Package (SAS Institute, Cary, NC). Shapiro-Wilk test was applied to test for normality. Comparisons between the groups were performed using a Two-Way Analysis of Variance (ANOVA) with the inclusion of an interaction term between smoking status and physical activity level. To assess effect of body weight and food intake, a 2-Way ANOVA was performed with time and group being the independent variable. A Tukey-Kramer *post hoc* test was used for multiple comparisons. Spearman's rho was used to assess correlations between muscle cross-sectional area and bone parameters. P values less than 0.05 were considered significant. Data are expressed as means ± standard deviation.

Legends Online Supplement Figure

Figure E1. Effects of cigarette smoke exposure and increased levels of physical inactivity on maximal exercise capacity. Smoking did not affect running speed. Running speed was reduced in extremely inactive mice (E) after 24 weeks, while it was increased in the active (Ac) and inactive (I) groups. Values are means ± SD. * $p<0.0001$ E versus others

Figure E2. Effects of cigarette smoke exposure and increased levels of physical inactivity on latency-to-fall time. Even though latency-to-fall time was not affected by smoking, it was reduced in the inactive (I) and extremely inactive mice (E) after 24 weeks compared to the active (Ac) mice. Values are means ± SD. § $p<0.0001$ E versus Ac; ** $p<0.05$ I versus E; *** $p<0.01$ I versus Ac.

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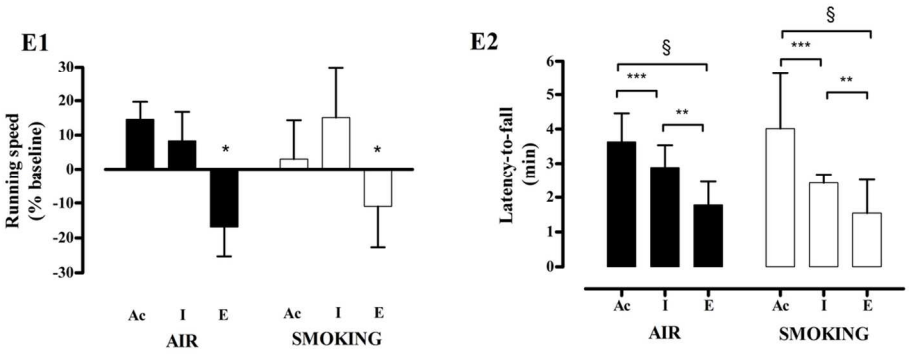

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